

Fig. 4. Marginal and scaled distribution for (A) the SST trend time series and (B) the detrended SST variability time series. Values greater than one (the vertical line in the color bar at the bottom) account for the MI shared between the variables.

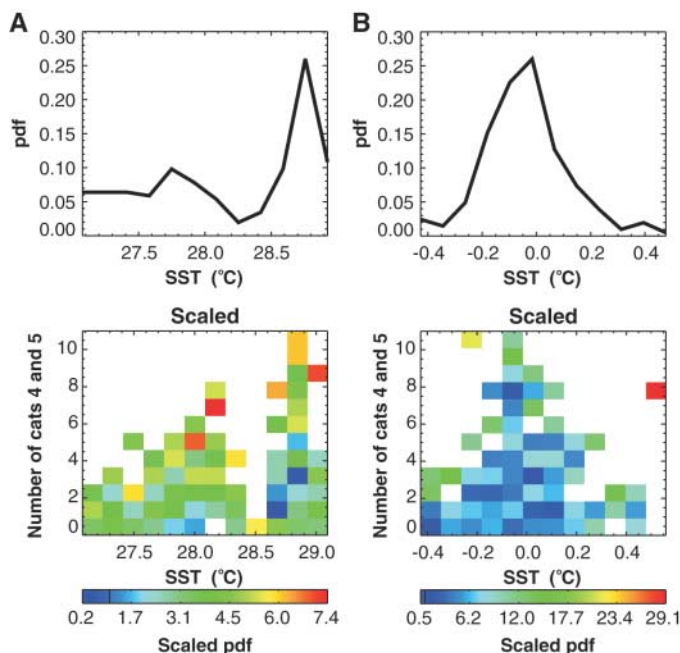


Table 2. Correlations for individual ocean basins between NCAT45 and variables for which there is a statistically significant trend in that basin, for the original time series in bold and the detrended time series in parentheses. NA, not applicable.

Variable	EPAC	NATL	SPAC
SST	0.32* (0.06)	0.67* (0.20)	0.29 (−0.10)
Specific humidity	0.23 (0.13)	NA	NA
Wind shear	NA	−0.61* (−0.45)*	NA
Stretching deformation	NA	NA	−0.67* (−0.55)*

*Statistically significant correlations at the 95% confidence level.

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- Values of MI vary from 0 (total independence) to 2.8 (total dependence), corresponding to the entropy of NCAT45 (minimum among all variables).
- SST correlations for the remaining three basins are as follows (using the same notation as in Table 2): WPAC **0.44*** (0.13), NIO **0.28** (−0.10), SIO **0.54*** (0.02).
- This research was supported by the Climate Dynamics Division of NSF under award NSF-ATM 0328842.

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Evolution of Hormone-Receptor Complexity by Molecular Exploitation

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According to Darwinian theory, complexity evolves by a stepwise process of elaboration and optimization under natural selection. Biological systems composed of tightly integrated parts seem to challenge this view, because it is not obvious how any element's function can be selected for unless the partners with which it interacts are already present. Here we demonstrate how an integrated molecular system—the specific functional interaction between the steroid hormone aldosterone and its partner the mineralocorticoid receptor—evolved by a stepwise Darwinian process. Using ancestral gene resurrection, we show that, long before the hormone evolved, the receptor's affinity for aldosterone was present as a structural by-product of its partnership with chemically similar, more ancient ligands. Introducing two amino acid changes into the ancestral sequence recapitulates the evolution of present-day receptor specificity. Our results indicate that tight interactions can evolve by molecular exploitation—recruitment of an older molecule, previously constrained for a different role, into a new functional complex.

The ability of mutation, selection, and drift to generate elaborate, well-adapted phenotypes has been demonstrated theoretically (1, 2), by computer simulation (3, 4), in the laboratory (5, 6), and in the field (7). How evolutionary processes assemble complex systems that depend on specific interactions among

the parts is less clear, however. Simultaneous emergence of more than one element by mutational processes is unlikely, so it is not apparent how selection can drive the evolution of any part or the system as a whole. Most molecular processes are regulated by specific interactions, so the lack of exemplars for the emergence of

such systems represents an important gap in evolutionary knowledge. As Darwin stated, “If it could be demonstrated that any complex organ existed which could not possibly have been formed by numerous, successive, slight modifications, my theory would absolutely break down” (8).

The functional interaction between the steroid hormone aldosterone and its specific partner the mineralocorticoid receptor (MR)—a ligand-activated transcriptional regulator (9, 10)—illustrates this evolutionary puzzle. MR and the glucocorticoid receptor (GR) descend from a gene duplication deep in the vertebrate lineage (11) and now have distinct signaling functions. In most vertebrates, GR is specifically activated by the stress hormone cortisol to regulate metabolism, inflammation, and immunity (9). MR is activated by aldosterone to control electrolyte homeostasis and other processes (9, 12). MR can also be activated by cortisol, although the presence of a cortisol-

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clearing enzyme in many MR-expressing tissues makes the receptor a largely aldosterone-specific factor (12). It is not obvious how the tight aldosterone-MR partnership could have evolved. If the hormone is not yet present, how can selection drive the receptor's affinity for it? Conversely, without the receptor, what selection pressure could guide the evolution of the ligand?

To reconstruct the evolution of the MR's interaction with aldosterone, we characterized the functions of the ancestral corticoid receptor (AncCR)—the ancient protein from which GR and MR descend by gene duplication. To improve the robustness of this inference, we first isolated corticoid receptor sequences from basal vertebrate taxa. Using the polymerase chain reaction (13), we identified a single corticoid receptor in jawless fishes—the lamprey *Petromyzon marinus* and the hagfish *Myxine glutinosa*—and both GR and MR in an elasmobranch, the skate *Raja erinacea*. Phylogenetic analysis indicates that the duplication leading to GR and MR occurred >450 million years ago, after the divergence of jawless fishes but before the split of cartilaginous fish from bony vertebrates (Fig. 1 and supplementary figs. S1 to S3). Functional assays (13) indicate that the basal receptors are activated by very low doses of aldosterone, cortisol, and 11-deoxycorticosterone (DOC); they are similar in this respect to MRs of tetrapods and teleosts (Fig. 2 and figs. S4 and S5) (14–16). The only receptors insensitive to aldosterone are the GRs of tetrapods and teleosts.

Given these results, the most parsimonious scenario is that AncCR was capable of being activated by aldosterone and that aldosterone sensitivity was lost in the GRs of bony vertebrates (Fig. 1). To test this hypothesis, we used gene resurrection (17) to experimentally characterize the ancestral CR. On the basis of the ML phylogeny and a large alignment of extant receptor sequences (table S1), we inferred the maximum likelihood (ML) amino acid sequence of AncCR's ligand-binding domain (LBD), the functionally separable region that contains the protein's ligand-regulated transcriptional functions (13). The ancestral sequence was inferred with strong support: The mean posterior probability (PP) was 94%, and two-thirds of sites had PP > 99% (table S2). AncCR-LBD is most similar to aldosterone-activated receptors MRs and CRs and differs from them by just one residue in the ligand-binding pocket (table S3).

We synthesized the AncCR-LBD sequence and expressed it in cultured cells; using a reporter assay, we found that AncCR is a sensitive and effective aldosterone receptor (13). Like the extant CRs and MRs, it is also activated by low doses of DOC and, to a lesser extent, cortisol (Fig. 3A). AncCR's aldosterone sensitivity is robust to uncertainty about the phylogeny and stochastic error in the sequence reconstruction. We used Bayesian phylogenetic

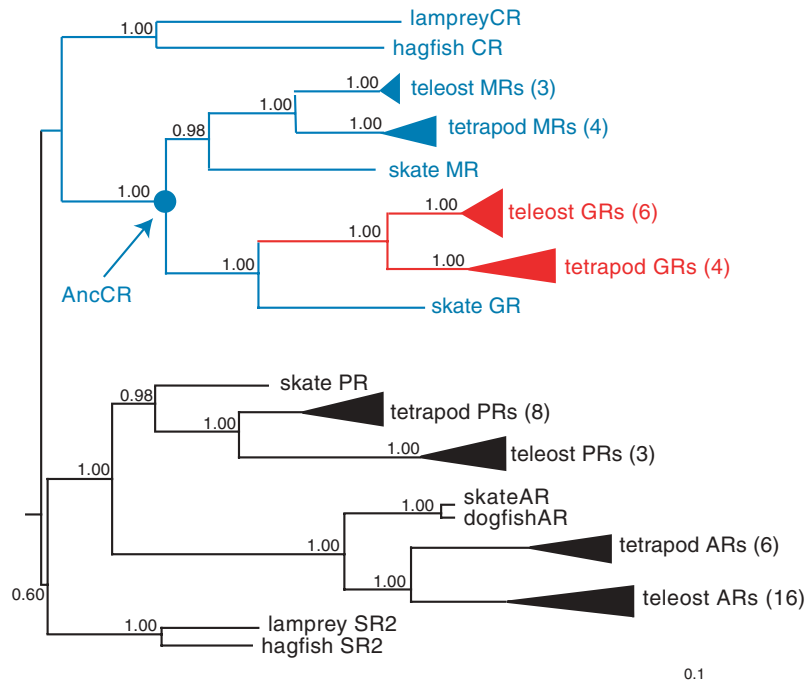


Fig. 1. Phylogeny of steroid hormone receptors. The gene family tree of 59 steroid and related receptor amino acid sequences was inferred using maximum likelihood (ML), Bayesian Markov chain Monte Carlo (BMCMC), and maximum parsimony (13). ML branch lengths and BMCMC posterior probabilities for major nodes are shown. The number of sequences in each clade is in parentheses. The ancestral corticoid receptor (AncCR) is marked with a circle. Blue, aldosterone-activated receptors; red, aldosterone-insensitive glucocorticoid receptors; black, noncorticoid receptor outgroups. For complete phylogenies and sequences, see figs. S1 to S3 and table S1.

ics to collect a large sample of plausible trees and reconstructed AncCR-LBD on all 467 trees in the 95% credible set; the ancestral sequence on every tree was identical to that on the ML tree, except for one site. When the alternate state was introduced into the reconstructed protein, AncCR became even more sensitive to aldosterone (fig. S6). To characterize AncCR's robustness to stochastic error, we examined positions that had an alternate state with PP > 0.20. In all cases but one, the alternate state is found in other aldosterone-activated receptors and is therefore not sufficient to abolish aldosterone sensitivity; introducing the exception into AncCR had no effect on ligand-activation (fig. S6). Finally, among sites that make contact with the ligand in the MR crystal structure (18), only one was ambiguously reconstructed. The mutagenized AncCR with the alternate state remained highly sensitive to aldosterone (fig. S6).

The aldosterone activation of AncCR—like that of the agnathan, elasmobranch, and teleost receptors—is surprising, because aldosterone has long been considered a tetrapod-specific hormone. Using a very sensitive fractionation and immunodetection strategy (13), we confirmed that aldosterone is absent from the plasma of lamprey and hagfish (Fig. 3B). Further, when interrenal gland explants were incubated with appropriate precursors and stimulatory hormones, neither species produced aldosterone (fig. S7). Aldosterone has been reliably detected

in tetrapods (9), but is absent from teleosts (19), elasmobranchs (20, 21), and agnathans, as our experiments show. The capacity to synthesize aldosterone therefore evolved relatively recently, in the lineage leading to tetrapods. Aldosterone's emergence was due to modification of cytochrome P-450 11 β -hydroxylase, the ancestral function of which is to hydroxylate DOC in glucocorticoid synthesis, a function present in all jawed vertebrates. Only in tetrapods has this enzyme evolved the additional capacity to hydroxylate corticosterone, allowing aldosterone synthesis (Fig. 4A) (19, 22, 23).

The sensitivity of corticoid receptors to aldosterone is therefore more ancient than the hormone itself (Fig. 4B). AncCR must have been regulated by a different ligand; one candidate is DOC, which is produced by agnathans (24) and by all jawed vertebrates as an intermediate in the synthesis of other corticosteroids (Fig. 4A). AncCR and the agnathan CRs, like the MRs of tetrapods and teleosts (15, 16), are very sensitive to DOC (Fig. 3A and fig. S4). Whatever the precise identity of the ancestral ligand, AncCR's aldosterone responsiveness, like that of CRs and MRs in species that do not produce the hormone, is due to aldosterone's structural similarity to steroids that do activate the receptor. Aldosterone differs from DOC only by small moieties at the 18- and 11-positions; our experiments show that neither of these affects activation of the ancestral or extant CRs.

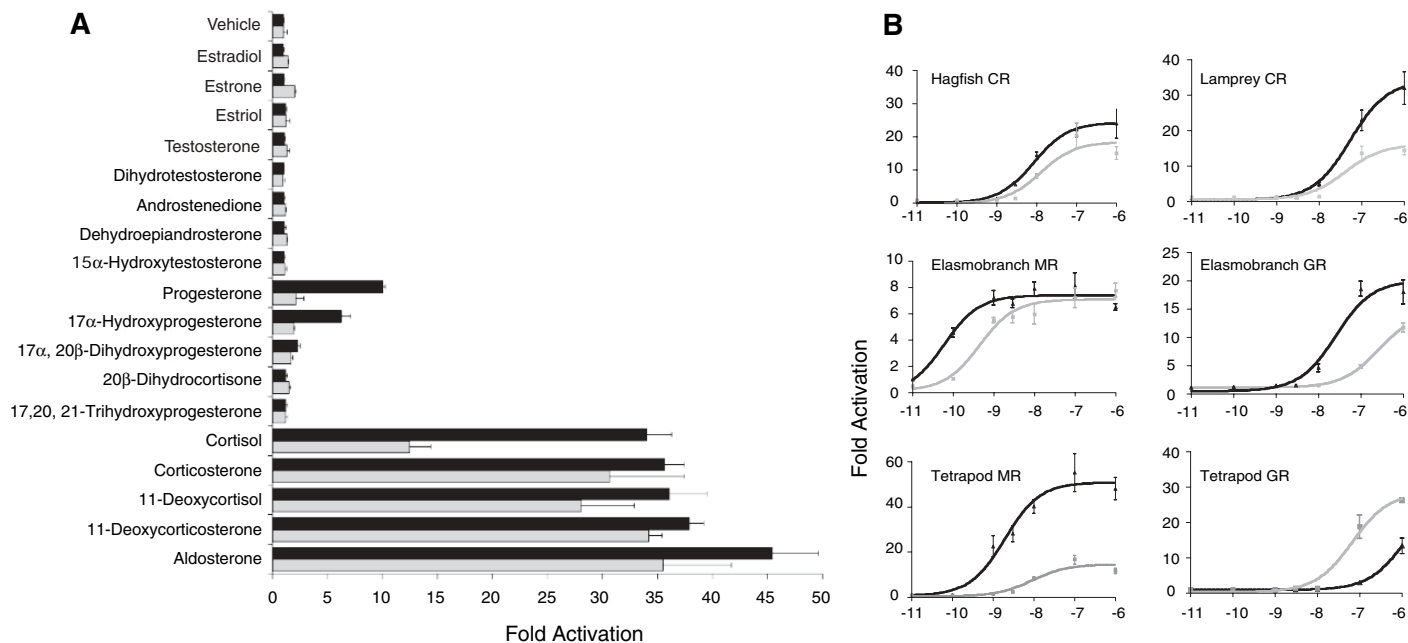


Fig. 2. Corticoid receptors (CRs) from basal vertebrates are activated by aldosterone. **(A)** Activation of a luciferase reporter gene by CR LBDs of hagfish (black) and lamprey (gray) with 100 nM hormone. Fold-activation indicates reporter activity compared with treatment with vehicle only; error bars are SEM for three replicates. **(B)** Dose-response for reporter expression by various CR-LBDs with aldosterone (black) or cortisol (gray). Full-length receptors expressed in different cell types show similar results (see fig. S5).

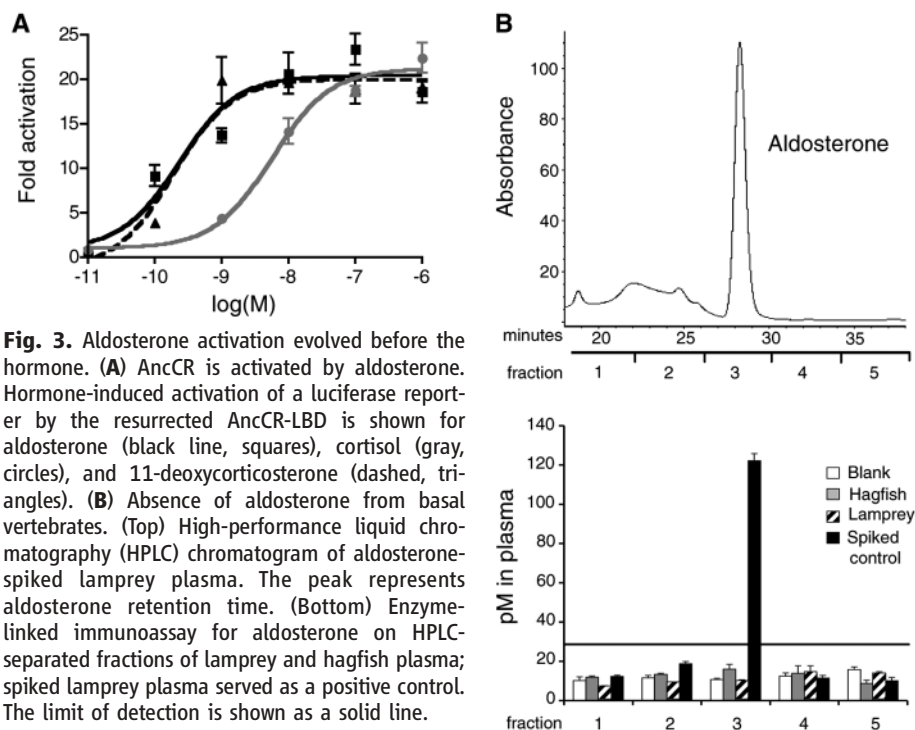


Fig. 3. Aldosterone activation evolved before the hormone. **(A)** AncCR is activated by aldosterone. Hormone-induced activation of a luciferase reporter by the resurrected AncCR-LBD is shown for aldosterone (black line, squares), cortisol (gray, circles), and 11-deoxycorticosterone (dashed, triangles). **(B)** Absence of aldosterone from basal vertebrates. (Top) High-performance liquid chromatography (HPLC) chromatogram of aldosterone-spiked lamprey plasma. The peak represents aldosterone retention time. (Bottom) Enzyme-linked immunoassay for aldosterone on HPLC-separated fractions of lamprey and hagfish plasma; spiked lamprey plasma served as a positive control. The limit of detection is shown as a solid line.

Extant MRs retain the ancestral phenotype, so the specificity of the MR-aldosterone relationship is due to the secondary loss of aldosterone sensitivity in the GR (Fig. 4B). To understand the mechanistic basis for this functional shift,

we identified sequence changes that are phylogenetically and functionally diagnostic, defined as having occurred on the branch where aldosterone sensitivity was lost, with one state conserved in all the aldosterone-activated re-

ceptors and a different state in all aldosterone-insensitive GRs. We introduced all four single GR-diagnostic states and all six twofold combinations into AncCR-LBD using mutagenesis and determined their effect on receptor function. One combination—replacement of Ser¹⁰⁶ with Pro (S106P) and Leu¹¹¹ with Gln (L111Q) (numbered by position in AncCR-LBD)—conferred a GR-like phenotype: The receptor's median effective concentration (EC₅₀) for aldosterone increased by three orders of magnitude, but moderate cortisol and DOC sensitivity were retained (Fig. 4C). None of the other mutants showed this pattern (table S4). Structural studies of the human GR have shown that these two residues change the architecture of the ligand-binding pocket and alter contacts with steroid in ways that exclude aldosterone and facilitate cortisol-activation (18, 25). Our data thus indicate that the aldosterone specificity of MR has a simple and conserved mechanistic basis—two crucial replacements in the GRs that wiped out ancestral sensitivity to aldosterone.

To reconstruct the trajectory of GR sequence evolution, we introduced each replacement in isolation and found that both are required to yield the GR phenotype. L111Q alone radically reduces activation by all ligands tested (Fig. 4C). In contrast, S106P reduces aldosterone and cortisol sensitivity, but this receptor remains highly DOC-sensitive. In the S106P background, L111Q further reduces aldosterone sensitivity but now restores cortisol response to levels character-

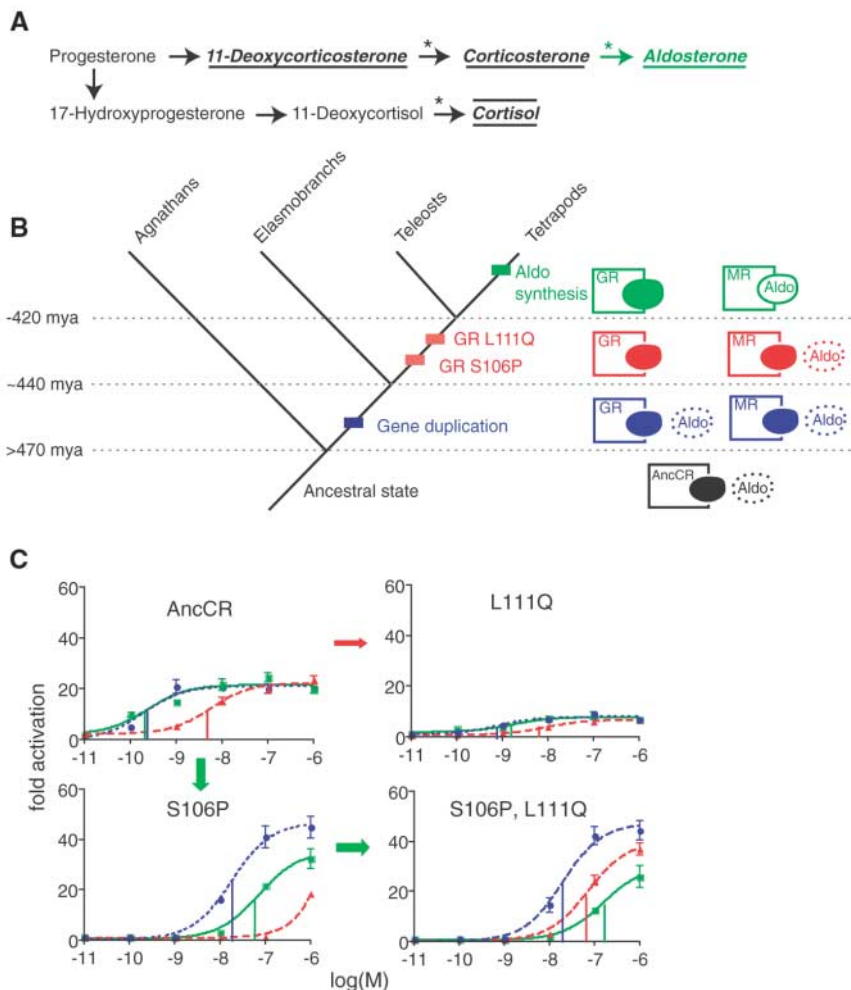


Fig. 4. Evolution of specific aldosterone-MR signaling by molecular exploitation. **(A)** Synthesis pathway for corticosteroid hormones. Ligands for the ancestral CR and extant MRs are underlined; cortisol, the ligand for the tetrapod GR, is overlined. The terminal addition of aldosterone is in green. Asterisks, steps catalyzed by the cytochrome P-450 11 β -hydroxylase enzyme; only the tetrapod enzyme can catalyze the step marked with a green asterisk. **(B)** MR's aldosterone sensitivity preceded the emergence of the hormone. The vertebrate ancestor did not synthesize aldosterone (dotted circle), but it did produce other corticosteroids (filled circle); it had a single receptor with affinity for both classes of ligand. A gene duplication (blue) produced separate GR and MR. Two changes in GR's sequence (red) abolished aldosterone activation but maintained cortisol sensitivity [see (C)]. In tetrapods, synthesis of aldosterone emerged due to modification of cytochrome P-450 11 β -hydroxylase. mya, million years ago. **(C)** Mechanistic basis for loss of aldosterone sensitivity in the GRs. Phylogenetically diagnostic amino acid changes that occurred during GR evolution were introduced into AncCR-LBD by mutagenesis. Dose-response is shown for aldosterone (green), DOC (blue), and cortisol (red). The double mutant (bottom right) has a GR-like phenotype. Arrows show evolutionary paths via a nonfunctional (red) or functional (green) intermediate.

istic of extant GRs. A mutational path beginning with S106P followed by L111Q thus converts the ancestor to the modern GR phenotype by functional intermediate steps and is the most likely evolutionary scenario (26).

Our findings demonstrate that the MR-aldosterone partnership evolved in a stepwise fashion consistent with Darwinian theory, but the functions being selected for changed over time. AncCR's sensitivity to aldosterone was present before the hormone, a by-product of selective constraints on the receptor for activation by its native ligand. AncCR and its descendant genes

were structurally preadapted for activation by aldosterone when that hormone evolved millions of years later. After the duplication that produced GR and MR, only two substitutions in the GR lineage were required to yield two receptors with distinct hormone-response profiles. The evolution of an MR that could be independently regulated by aldosterone enabled a more specific endocrine response, because it allowed electrolyte homeostasis to be controlled without also triggering the GR stress response, and vice versa.

This evolutionary scenario—recruiting an ancient receptor into partnership with a novel

ligand—is the obverse of the dynamic previously established for the androgen and progesterin receptors (AR, PR). In that case, duplicates of an ancient estrogen-responsive receptor evolved affinity for steroids that previously served as intermediates in estrogen synthesis (11, 27). Together, the hormone-first history of AR and PR and the receptor-first history of MR point to a general evolutionary dynamic: Novel interactions emerge when a newly generated molecule—usually a slight structural modification or duplicate of an existing one—recruits as a binding partner a more ancient molecule, which was previously constrained by selection for an entirely different function. This model, which we call “molecular exploitation,” is consistent with findings that other ancient biological features have been co-opted for novel functions (28–30).

The puzzle that complex systems pose for Darwinian evolution depends on the premise that each part has no function—and therefore cannot be selected for—until the entire system is present. This puzzle might indeed cause Darwin's theory to “break down” if the functions of the parts must remain static for all time. But virtually all molecules can and do participate in more than one process or interaction, so a complex's elements may have been selected in the past for unrelated functions. Our work indicates that tightly integrated systems can be assembled by combining old molecules with different ancestral roles together with new ones—generated by gene duplication or elaboration of enzymatic pathways—that represent slight structural variants on older elements. We propose that molecular exploitation will be a predominant theme in evolution, one that may provide a general explanation for how the molecular interactions critical for life's complexity emerged in Darwinian fashion.

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Phylogeny of the Ants: Diversification in the Age of Angiosperms

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We present a large-scale molecular phylogeny of the ants (Hymenoptera: Formicidae), based on 4.5 kilobases of sequence data from six gene regions extracted from 139 of the 288 described extant genera, representing 19 of the 20 subfamilies. All but two subfamilies are recovered as monophyletic. Divergence time estimates calibrated by minimum age constraints from 43 fossils indicate that most of the subfamilies representing extant ants arose much earlier than previously proposed but only began to diversify during the Late Cretaceous to Early Eocene. This period also witnessed the rise of angiosperms and most herbivorous insects.

Ants are a ubiquitous and dominant feature of the terrestrial landscape, playing key roles in symbiotic interactions, soil aeration, and nutrient cycling. They have a rich fossil record (1), yet the evolutionary history of the ~11,800 described modern species remains poorly resolved.

Bolton's (2) recent revision of ants (Hymenoptera: Formicidae) recognized 288 genera in 21 [subsequently reduced to 20 (2, 3)] subfamilies. Several phylogenies have been proposed based primarily on morphological characters, but these reflect disagreement about the positions of major lineages (fig. S2) (4–7). Recent molecular analyses have included only a moderate number of taxa and recovered only weak support for most clades (8, 9), although more comprehensive studies are under way (10).

To evaluate competing phylogenetic hypotheses, we analyzed 4.5 kb of sequence data (Fig. 1) from portions of five nuclear genes and one mitochondrial gene from 139 ant genera and six Aculeata Hymenoptera outgroups ($n = 149$ specimens) representing 19 of the 20 currently recognized extant subfamilies. The only ant subfamily not included was Aenictogitoninae, a rare group known only from males collected at lights in equatorial Africa. The monophyly of the Formicidae itself was strongly supported in all analyses (Table 1).

Analyses with several methods (11) resulted in a well-resolved phylogeny that divided the family into three groups: the leptanilloid clade, a basal lineage containing 1

subfamily (Leptanillinae) and sister to all other ants; the poneroid clade, containing 5 subfamilies (Agroecomyrmecinae, Amblyoponinae, Paraponerinae, Ponerinae, and Proceratiinae); and the formicoid clade, containing the remaining 13 subfamilies sampled in this study. All three clades were supported by 100% Bayesian posterior probability (bpp) support, but only the formicoid and leptanilloid clades were well supported in the maximum likelihood analyses [$\geq 94\%$ maximum likelihood bootstrap (ml bs)].

Of the 19 subfamilies investigated here, 14 were recovered as monophyletic with strong support, and none of the three monotypic taxa, each represented by a single extant species (Agroecomyrmecinae, Aneuretinae, and Paraponerinae), nested within another lineage, validating their status as separate subfamilies. However, the three sampled genera of Cerapachyinae were paraphyletic in all analyses. The eight genera of Amblyoponinae grouped together in a clade that lacked support, although the monophyly of Amblyoponinae genera was well supported in an earlier molecular study (2).

The monophyly of the Leptanillinae was strongly supported (100% bpp and ml bs), and its basal position was recovered in all analyses. Ward (6) noted that a basal position of *Leptanilla* within the poneroid group implies that tergo-sternal fusion of abdominal segments III and IV in the worker caste occurred early in ant evolution and was lost secondarily in many lines. Our results indicate that these characters are indeed labile and homoplasious. Although the basal position of Leptanillinae was suggested in other molecular studies (2, 10), previous phylogenetic hypotheses based on morphology had failed to place it in a basal position among extant ants.

Bolton (2) proposed a "poneromorph" clade, including Amblyoponinae, Ectatomminae, Het-

eroponerinae, Paraponerinae, Ponerinae, and Proceratiinae; our results exclude Ectatomminae and Heteroponerinae but add Agroecomyrmecinae. The latter is represented by a single extant species, *Tatuvidris tatusia*, and two fossil genera, and its placement within the poneroid clade is entirely novel. Both Ectatomminae and Heteroponerinae nested within the formicoid clade. Although the poneroid clade received less support in the maximum likelihood and maximum parsimony analyses (Table 1), it was strongly supported in the Bayesian analysis (100% bpp). It seems likely that the five included subfamilies form a monophyletic group or, alternatively, a basal polytomy, but in either case they remain outside both the leptanilloid and the formicoid clades.

The inclusion of Heteroponerinae within the formicoid clade is also unexpected. As suggested by their name, heteroponerines have historically been placed in the poneromorph clade. Moreover, Ectatomminae, until recently also considered poneromorphs, appear to be closely related to Heteroponerinae. These findings, combined with the lack of stability for the "poneromorphs" observed in morphological analyses (4–7), underscore the extent to which our understanding of ancestral ant morphology and behavior must be revised.

The phylogenetic position of *Aneuretus*, today restricted to Sri Lanka, has been hypothesized to be basal either to the Dolichoderinae or to the Dolichoderinae + Formicinae (12, 13). We recover *Aneuretus* as basal to the Dolichoderinae, with both groups separated from Formicinae, implying that the sting has been reduced independently at least twice in the ants (Dolichoderinae and Formicinae).

The ant fossil record is extensive, with more than 60 extant and 100 extinct genera. The oldest reliably dated fossils are ~100 million years (My) old, from Early Cretaceous French and Burmese ambers (14, 15). These include both *Gerontiformica* and *Burmomyrma* (Aneuretinae), with features typical of modern "crown group" ants, as well as Sphecomyrminae, with features typical of basal "stem group" ants. Although no older sphecomyrminae are known, the presence of stem and crown group ants in these roughly coeval ambers implies an earlier history of Formicidae. The status of the Armaniinae/-idae as stem group ants is controversial (1, 3, 15), but if they are viewed as sister to Formicidae, this also implies an extension of the minimum age of ants to the maximum age of Armaniinae/-idae, which has been estimated to be ~125 My (16).

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